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Point of VIEW

Glucose: A Novel Regulator of Notch Signaling

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ABSTRACT Notch signaling regulates cell fate during the development of many tissues. A new *Drosophila* mutant, *rumi*, is defective in Notch signaling because it cannot add glucose to serine in epidermal growth factor repeats of Notch extracellular domain. This is the first biological role for glucose covalently attached to a cell surface signaling receptor.

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Drosophila Notch is an ~300 kDa glycoprotein expressed at the cell surface as a noncovalent heterodimer with a single transmembrane domain. The *Drosophila* Notch ligands Delta and Serrate are also cell surface transmembrane glycoproteins. Notch signaling occurs when Notch ligand(s) bind to the extracellular domain (ECD) of Notch in an apposing cell. Ligand binding, and thus Notch signaling, is regulated by glycans covalently attached through *O*-linked fucose to epidermal growth factor (EGF) repeats of Notch ECD (*1, 2*). A recent paper by Acar *et al.* in *Cell* (*3*) reveals that another type of glycan attached to Notch EGF repeats *via O*-linked glucose is also critical for Notch signaling (Figure 1). Interestingly, while Notch lacking *O*-glucose cannot signal at 25 °C, leading to death at the larval stage, at 18 °C mutant flies have very mild Notch signaling defects.

The existence of *O*-glucose glycans on Notch was first discovered in biochemical analyses of mammalian Notch1 (*4*). The glucose is attached directly to serine between the first and second cysteine residues of EGF repeats within the consensus sequence C^1-X-S-X-P-C^2. In mammalian cells, the *O*-glucose is followed by a β1,3-xylose and then another xylose in β1,3-linkage to form the trisaccharide EGF-*O*-α1Glcβ1,3Xylβ1,3Xyl (*4*). *O*-Glucosylation of *Drosophila* Notch was investigated by Acar *et al.* (*3*) using LC-MS/MS analysis of tryptic peptides from Notch ECD (EGF7 to the transmembrane domain) prepared from *Drosophila* S2 cells. Glucosylated peptides containing the expected consensus sequence for *O*-glucosylation were readily identified and found to contain glucose. However, none contained xylose. This may be because *O*-glucose is not further extended in *Drosophila* Notch or, perhaps more likely, because the appropriate glycosyltransferase(s) are not expressed by S2 cells. For example, S2 cells do not express Fringe, the β1,3GlcNAc-transferase that transfers GlcNAc to *O*-fucose on Notch ECD in *Drosophila* (*1*).

An *O*-glucosyltransferase activity that acts on EGF repeats was identified several years ago in mammalian cells and tissues (*5*). However, the corresponding gene eluded bioinformatics searches. It was discovered by Acar *et al.* (*3*) in a genetic screen designed to isolate temperature-sensitive mutants of *Drosophila* affected in Notch signaling. One complementation group called *rumi*, for a 13th-century Persian/Turkish poet, was mutated in a gene encoding a CAP 10 domain that is often associated with bacterial glycosyltransferases. Flies with a null *rumi* mutation, which completely lack *O*-glucosyltransferase activity as well as Rumi protein, have defects in Notch signaling that manifest strongly at temperatures >25 °C but very mildly, if at all, at 18 °C. A temperature-sensitive Notch signaling phenotype is also observed in a GDP-fucose transporter mutant, which presumably affects the transfer of *O*-fucose to Notch (*6*). The mildness of this phenotype strongly indicates the existence of an alternative means of GDP-fucose transport, because complete loss of GDP-fucose from the secretory pathway should result in the same severe Notch signaling defects observed in flies lacking Ofut1 (*7, 8*). Structural analyses of tryptic peptides obtained from

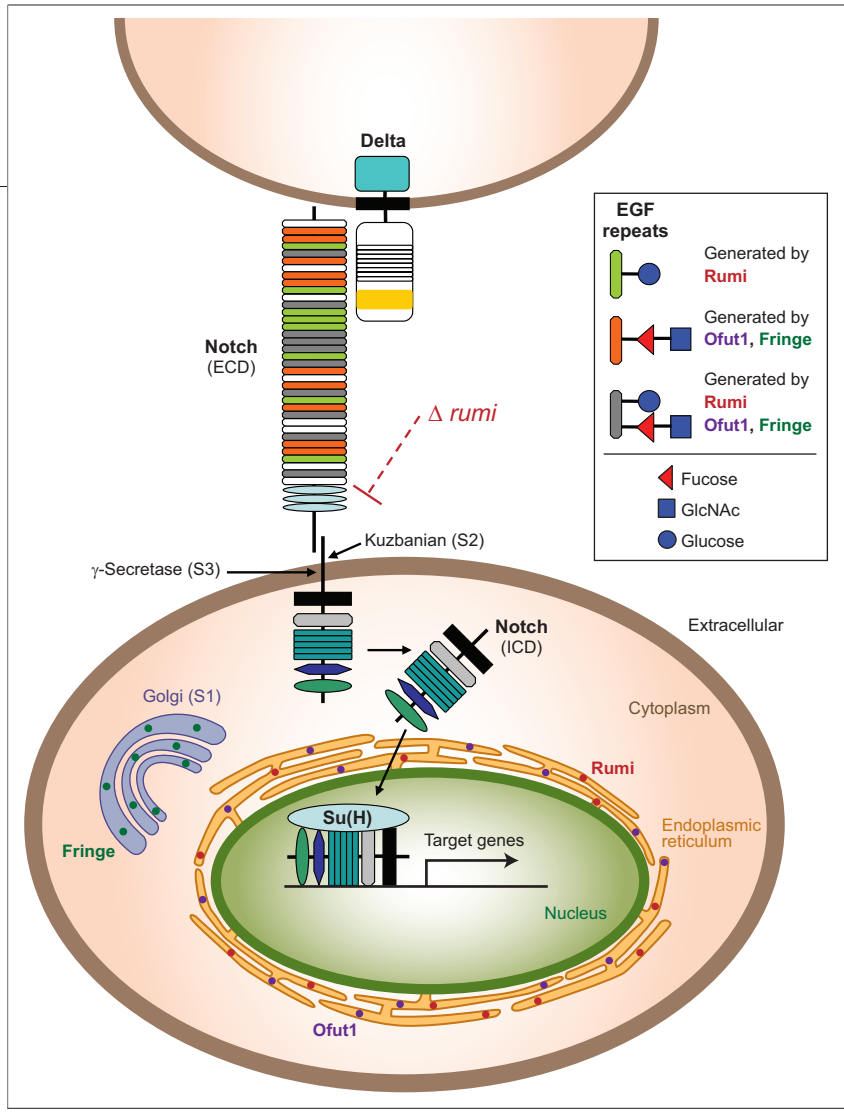

Figure 1. Notch biosynthesis. The biosynthesis of Notch begins in the ER, where EGF repeats of the ECD are cotranslationally modified with *O*-glucose by the action of Rumi and with *O*-fucose by the action of Ofut1. Rumi (Poglut) and Ofut1 are both soluble glycosyltransferases with a C-terminal ER retention signal. Notch in the ER is a type I transmembrane glycoprotein of ~300 kDa. As it passes through the Golgi, Notch may be cleaved by furin (S1 cleavage) to form a noncovalently associated heterodimer and modified by Fringe. At the plasma membrane, Notch ECD interacts with Delta or Serrate ligand in an adjacent cell. Ligand binding induces a conformational change that allows the metalloprotease Kuzbanian to cleave Notch (S2 cleavage) at a point just outside the transmembrane domain. The S2 cleavage releases Notch ECD, which is endocytosed by the ligand-expressing cell. The S2 cleavage is followed by a cleavage within the transmembrane region by a complex that includes γ-secretase. The S3 cleavage releases Notch ICD, which translocates to the nucleus and in combination with Suppressor-of-hairless (Su(H)) and other transcriptional activators induces the expression of target genes, including Hairy/Enhancer-of-Split (Hes). In a *rumi* null mutant, Notch is not *O*-glucosylated in the ER. The consequence for Notch signaling is that at temperatures >25 °C, Notch signaling is generally and severely impaired. Notch lacking *O*-glucose on EGF repeats reaches the cell surface and is bound by Delta (or Serrate) but is not induced to adopt the conformation required for the S2 cleavage. This precludes the release of Notch ICD and the transduction of a Notch signal. Reprinted in part from *Current Opinion in Structural Biology*, *17*, Stanley, P., Regulation of Notch signaling by glycosylation, 530–535, Copyright 2007, with permission from Elsevier.

Notch EGF repeats synthesized in S2 cells in which Rumi was knocked down by short-hairpin RNA showed loss of *O*-glucose by ~90–95% compared with control S2 cells. Thus, there is no alternative activity to Rumi in S2 cells. However, it will be important to structurally characterize Notch EGF repeats from *rumi* null flies grown at 18 °C to determine whether they contain any *O*-glucose. Ideally, once sufficiently sensitive mass spectrometers become available, Notch from the different cell types affected by *rumi* mutations can be compared to determine whether there is any complementing activity *in vivo* that might cause transfer of *O*-glucose to Notch in the absence of Rumi.

Loss of Rumi activity affects Notch signaling at the level of the Notch ECD, upstream of the S2 cleavage induced by Kuzbanian (ADAM10 in mammals), and the subsequent intramembrane S3 cleavage by γ-secretase that releases Notch ICD. The latter cleavage, recently shown to occur most efficiently in endosomes (*9*), generates the Notch intracellular domain (ICD), which ultimately translocates to the nucleus and, in combination with transcriptional coactivators, activates target genes. The loss of the *O*-glucosyltransferase affects Notch S2 cleavage in a cell-autonomous manner. Notch signaling defects can be partially rescued in *rumi* mutant flies by adding an extra copy of Notch at 25 °C. On the other hand, Notch signaling defects may be readily observed at 18 °C in flies if they carry only one copy of Notch. On the basis of these and other genetic interaction experiments, it can be concluded that Rumi is a general regulator of Notch signaling. Interestingly, Notch ligands have EGF repeats that contain the consensus recognized by the *O*-glucosyltransferase but function normally in the absence of Rumi.

Notch-specific, cell-autonomous signaling defects also occur when Notch EGF repeats lack *O*-fucose, and this phenotype is not temperature-sensitive (*1*, *2*). Again, there are no apparent biological consequences for Notch ligands when they have no *O*-fucose glycans (*7*). The enzyme that transfers O-fucose to Notch EGF repeats is Ofut1 in *Drosophila* and Pofut1 in mammals. When overexpressed together with

Notch ECD in *Drosophila* S2 cells, Ofut1 and Notch ECD can be coimmunoprecipitated (*10, 11*), an indication that Ofut1 binds to Notch, presumably during synthesis in the endoplasmic reticulum (ER) (*10, 12*) and potentially during Notch trafficking to the cell surface (*11*). In the absence of Ofut1, *Drosophila* Notch accumulates intracellularly (*10, 11, 13*) and thus is not available for stimulation by Notch ligands at the cell surface. Interestingly, this is not the case when the Rumi *O*-glucosyltransferase (Poglut) is absent. Rumi, like Pofut1, has a KDEL ER retention sequence at its C-terminus. When this sequence is deleted, Rumi is secreted from S2 cells and cannot rescue Notch signaling in *rumi* mutants *in vivo*. Therefore, Rumi must be retained in the ER to functionally *O*-glucosylate Notch. Notch lacking *O*-glucose accumulates intracellularly somewhat at 30 °C but is also found robustly expressed at the cell surface. Therefore, unlike *Drosophila* Notch made in the absence of Ofut1, Notch made in the absence of Rumi is stably expressed at the cell surface. In addition, it binds Delta as shown by an *in vitro* binding assay. Moreover, binding of Delta to Notch ECD lacking *O*-glucose is markedly enhanced by the presence of Fringe at both 18 and 28 °C. Therefore, ligand binding to Notch made in the absence of Rumi is not impaired. This is in stark contrast to Notch ECD made in the absence of Ofut1, which does not bind to either Delta- or Serrate-expressing S2 cells (*10, 14, 15*).

A *rumi* mutant allele with a single point mutation (G189E) provided proof that it is the transferase rather than a chaperone activity of Rumi that regulates Notch signaling. *In vitro* enzyme assays revealed that Rumi/G189E has no *O*-glucosyltransferase activity. Western analyses from tissues of flies homozygous for this mutation showed that Rumi/G189E protein is expressed at the same level as wild-type Rumi *in vivo*. However, inactive Rumi cannot rescue Notch signaling. By contrast, an inactive form of Pofut1 (R245A) can at least partially rescue Notch signaling in an Ofut1 null background in *Drosophila* (*10, 13*) and in *Pofut1*$^{-/-}$ mouse embryonic stem cells (*16*). Thus, Ofut1 acts as a chaperone and a fucosyltransferase in the generation of active *Drosophila* Notch (*17, 18*), whereas Rumi appears to function solely as a glycosyltransferase.

In summary, the comprehensive analysis of *rumi* mutants (*3*) shows that the *Drosophila RUMI* gene encodes an *O*-glucosyltransferase that resides in the ER lumen and glucosylates Notch in EGF repeats that contain the consensus C^1-X-S-X-P-C^2. Loss of *O*-glucosylation does not appear to reduce cell surface Notch expression or its ability to bind Delta (and probably Serrate, though this was not tested directly). However, the S2 proteolytic activation of Notch normally induced by ligand binding does not occur at 28 °C. This strongly suggests, as concluded by Acar *et al*. (*3*), that Notch lacking *O*-glucose is improperly folded at 25 °C and cannot be induced by ligand binding into the appropriate conformation for S2 cleavage to occur. This in turn precludes S3 cleavage and thus Notch signaling. Consistent with this interpretation is the fact that, at 18 °C when folding may proceed more slowly, Notch lacking *O*-glucose signals almost as well as wild-type Notch. It will be most interesting to determine the phenotype of mammals lacking Rumi/Poglut. Given that mammals live at 37 °C, Notch signaling would be predicted to be severely compromised and perhaps exhibit a severe, global Notch phenotype as seen in mouse embryos lacking Pofut1 (*19*).

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